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Inhibition of actin polymerization in the NAc shell inhibits morphine-induced CPP by disrupting its reconsolidation

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Drug-associated contextual cues contribute to drug craving and relapse after abstinence, which is a major challenge to drug addiction treatment. Previous studies showed that disrupting memory reconsolidation impairs drug reward memory. However, the underlying mechanisms remain elusive. Although actin polymerization is involved in memory formation, its role in the reconsolidation of drug reward memory is unknown. In addition, the specific brain areas responsible for drug memory have not been fully identified. In the present study, we found that inhibiting actin polymerization in the nucleus accumbens (NAc) shell, but not the NAc core, abolishes morphine-induced conditioned place preference (CPP) by disrupting its reconsolidation in rats. Moreover, this effect persists for more than 2 weeks by a single injection of the actin polymerization inhibitor, which is not reversed by a morphine-priming injection. Furthermore, the application of actin polymerization inhibitor outside the reconsolidation window has no effect on morphine-associated contextual memory. Taken together, our findings first demonstrate that inhibiting actin polymerization erases morphine-induced CPP by disrupting its reconsolidation. Our study suggests that inhibition of actin polymerization during drug memory reconsolidation may be a potential approach to prevent drug relapse.

Drug addiction is a chronic brain disorder with a high rate of relapse, which is characterized by compulsive drug seeking and drug taking despite harmful consequences^{1,2}. It not only increases health care costs but also causes enormous social problems, for example, increased rates of disability and crime. However, even after prolonged abstinence, high relapse rates limit successful treatment of drug addiction³⁻⁵. The major cause of relapse is the robust and long-lasting memory formed by learned association between rewarding or aversive drug effects and drug-related environmental cues^{6,7}. Thus, it is urgent to develop a therapeutic approach to disrupt the associative memory between drug and drug-related cues. Memory reconsolidation sheds light on blocking this association to prevent contextual cue-induced relapse.

After learning, new memories are stabilized, named memory consolidation⁸. Then, retrieval of a memory trace induces an additional labile phase that requires an active process to restabilize the established memory, which is named reconsolidation⁹⁻¹⁴. Consistently, a short labile phase can be induced once consolidated drug-associated memories are retrieved, which provides a window to manipulate the established drug-associated memory.

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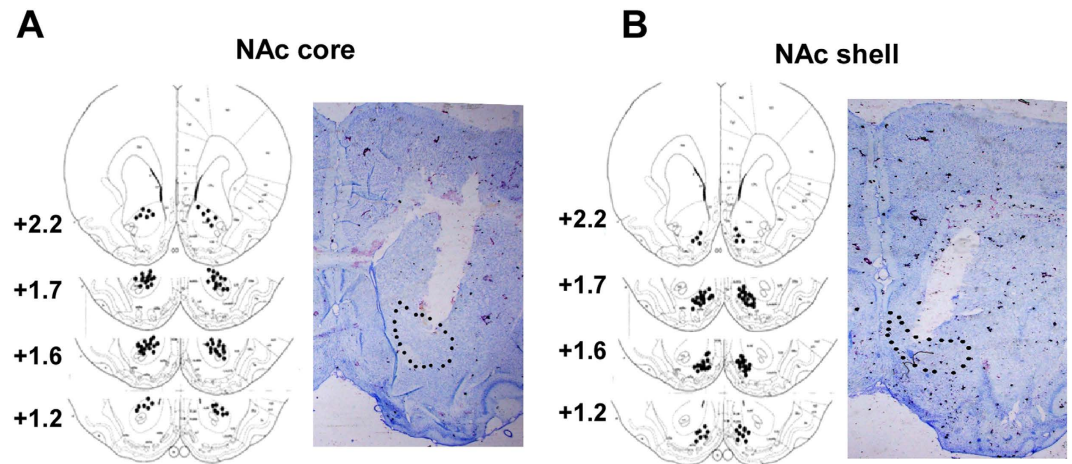


Figure 1. Schematic representation and photomicrographs of microinjection sites. (A) Distribution of microinjection sites in the NAc core (top panel) and the photomicrographs of representative sample punches and cannula placements in the NAc core (bottom panel). (B) Distribution of microinjection sites in the NAc shell (top panel) and the photomicrographs of representative sample punches and cannula placements in the NAc shell (bottom panel).

Although underlying mechanisms of drug memory reconsolidation remain elusive, increased evidence suggests that dysregulation of actin dynamics may play a key role in this process. First, actin is the primary structural component of cells and highly dynamic conversion between actin monomers (G-actin) and branched filaments (F-actin), i.e., actin rearrangements¹⁵, is essential to maintain cellular functions, particularly in neurons^{16,17}. Second, actin dynamics plays a crucial role in memory formation. For example, inhibition of actin polymerization by latrunculin A (Lat A) disrupts the late phase of long-term potentiation¹⁸. Rehberg *et al.* showed that actin filament arrest in the basolateral complex of the amygdala impairs fear memory consolidation and reconsolidation¹⁹. In addition, actin rearrangement is involved in drug-related memories^{20–22}. Moreover, systemic administration of rapamycin, an inhibitor of mTOR kinase, blocks morphine-induced CPP after re-exposure to morphine-paired environment cues, while rapamycin could inhibit actin reorganization²³. Above evidence suggests that actin dynamics may be involved in the reconsolidation of drug memories, e.g., morphine-induced CPP.

Nucleus accumbens (NAc) is a central component of the limbic system, which plays a major role in reward and addiction²⁴. The NAc consists of two main substructures, the core and shell, which are the inner and outer region of the NAc, respectively. Both of them are involved in reward and learning reinforcement. Previous studies suggest that they not only have distinct roles but also have overlap roles to certain extent in the above processes. First, reinstatement of drug-seeking behavior induced by re-exposure to heroin-associated environment cues or acute food deprivation stress requires the activation of D1-like receptors in the NAc shell but not that in the core^{25,26}. On the other hand, infusion of lactacystin, a proteasome inhibitor, into the core, but not the shell, prevents cocaine-associated memory extinction²⁷. Interestingly, microinjections of activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) antisense oligodeoxynucleotides into the NAc core impair the acquisition, expression and reinstatement of morphine-induced CPP, while intra-NAc shell injections only impair the expression of morphine-induced CPP²⁸. However, the role of the NAc core and shell in the reconsolidation of morphine-induced CPP and underlying mechanisms remain unknown.

In the present study, we report that inhibiting actin polymerization in the NAc shell, but not the NAc core, abolishes morphine-induced CPP by disrupting its reconsolidation. Moreover, this effect persists for more than 2 weeks by a single injection of the actin polymerization inhibitor, which is not reversed by a morphine-priming injection. Furthermore, the application of actin polymerization inhibitor outside the reconsolidation window has no effect on morphine-associated contextual memory. Our study suggests that inhibition of actin polymerization during drug memory reconsolidation may be a potential approach to prevent drug relapse.

Results

Inhibition of actin polymerization in the NAc core has no effect on the expression of morphine-induced CPP. After a pre-test of CPP, all rats received 8 days of CPP training, one session per day. The first CPP test was performed on day 9. On day 10, the rats received memory retrieval (i.e., re-exposure to the morphine-paired chamber for 10 minute) immediately followed by microinjections of vehicle or Lat A (an actin polymerization inhibitor) into the NAc core at the concentration of 0.5 $\mu\text{g}/\mu\text{l}$. On the next day, the CPP test (test 2) was performed (Figs 1A and 2A). Statistical analysis of CPP scores of rats receiving vehicle or Lat A microinjections revealed a significant effect of test phase ($F_{2,26} = 34.915$,

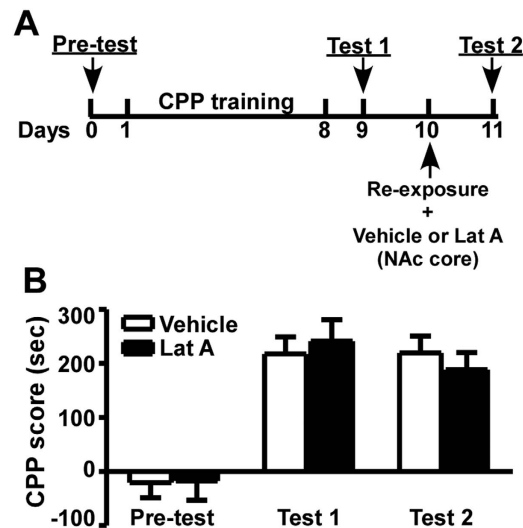


Figure 2. Inhibition of actin polymerization in the NAc core has no effect on the expression of morphine-induced CPP. (A) Timeline of the experimental procedure. (B) CPP scores of rats receiving microinjections into the NAc core. Values represent mean \pm SEM, $n = 7-8$ /group.

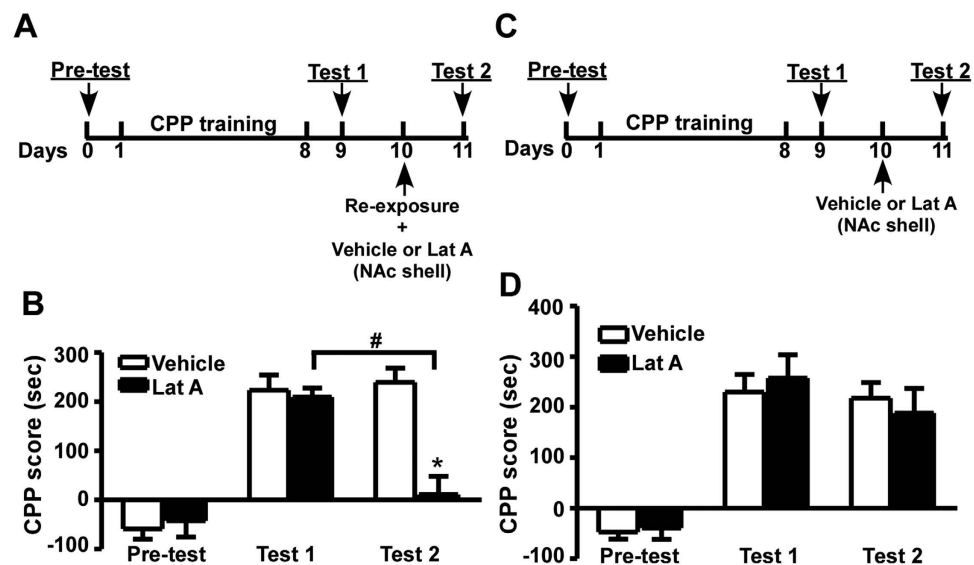


Figure 3. Inhibition of actin polymerization in the NAc shell inhibits morphine-induced CPP by disrupting its reconsolidation. (A) Timeline of the experimental procedure. (B) CPP scores of rats receiving microinjections into the NAc shell. (C) Timeline of the experimental procedure. Compared with (A), no re-exposure to the morphine-paired chamber was performed on day 9. (D) CPP scores of rats receiving microinjections into the NAc shell without re-exposure to the morphine-paired chamber. Values represent mean \pm SEM, $n = 7-8$ /group. * $P < 0.01$ vs. test 2 of the vehicle group and # $P < 0.01$ vs. test 1 of the Lat A group, two-way ANOVA with Tukey's post hoc test.

$P < 0.01$), while no significant effect was found between vehicle and Lat A treatments ($P > 0.05$) as well as from the interaction between treatment and test phase ($P > 0.05$) (Fig. 2B). These results demonstrate that inhibition of actin polymerization in the NAc core has no effect on the expression of morphine-induced CPP.

Inhibition of actin polymerization in the NAc shell inhibits morphine-induced CPP by disrupting its reconsolidation. To further investigate the effect of inhibition of actin polymerization in the NAc shell, the rats immediately received microinjections of vehicle or Lat A into the NAc shell instead of the NAc core after re-exposure to the morphine-paired chamber on day 10 (Figs 1B and 3A). A significant effect of test phase ($F_{2,26} = 32.646$, $P < 0.01$), treatment ($F_{1,13} = 17.905$, $P < 0.01$) and treatment \times test

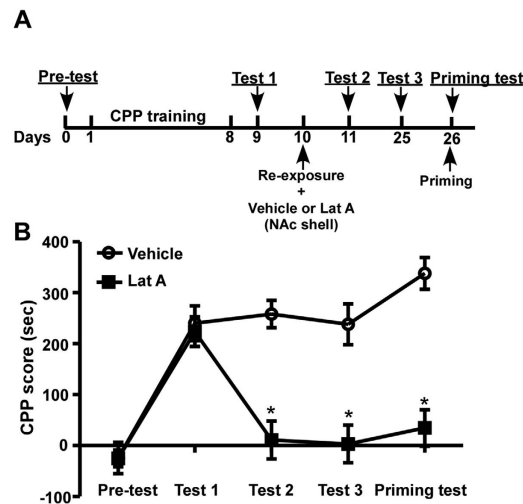


Figure 4. Inhibition of actin polymerization in the NAc shell has a long-term effect on disrupting morphine-induced CPP. (A) Timeline of the experimental procedure. (B) CPP scores of rats receiving microinjections into the NAc shell. Values represent mean \pm SEM, $n = 7-8$ /group. * $P < 0.01$ vs. each corresponding test of the vehicle group, two-way ANOVA with Tukey's post hoc test.

phase interaction ($F_{2,26} = 6.810$, $P < 0.01$) was revealed (Fig. 3B). Moreover, Tukey's post hoc analysis showed that a single Lat A injection completely inhibited the expression of morphine-induced CPP compared with vehicle treatment, 11.06 ± 37.24 s vs. 239.51 ± 28.55 s, $p < 0.01$ (Fig. 3B). These results indicate that a single Lat A microinjection into the shell inhibits the expression of morphine-induced CPP.

To investigate whether Lat A-induced disappearance of CPP expression is mediated by the disruption of its reconsolidation, the experiments without retrieval were performed as retrieval is an essential step of memory reconsolidation (Fig. 3C). The first 9-day procedure is as same as that shown in Fig. 3A. On day 10, the rats received microinjections of vehicle or Lat A into the NAc shell without re-exposure to the morphine-paired chamber. On day 11, the CPP test (test 2) was performed. As expected, Lat A had no significant effect on the expression of morphine-induced CPP compared with vehicle treatment ($P > 0.05$), although a significant effect of test phase ($F_{2,24} = 35.960$, $P < 0.01$) was maintained. In addition, the treatment \times test phase interaction was not significant ($F_{2,24} = 0.311$, $p > 0.05$) (Fig. 3D). These results demonstrate that retrieval is essential for Lat A-induced disappearance of CPP, indicating that the disruption of memory reconsolidation by Lat A contributes to the disappearance of CPP expression.

Inhibition of actin polymerization in the NAc shell has a long-term effect on disrupting morphine-induced CPP.

We have shown that a single post-retrieval microinjection of Lat A inhibited the expression of morphine-induced CPP (Fig. 3B). To further examine the long-term effect of Lat A treatment, morphine-induced CPP was re-tested after two weeks (test 3) (Fig. 4A). On day 26, the priming test of CPP was performed immediately after a priming injection of morphine. Statistical analysis revealed a significant effect of test phase ($F_{4,52} = 17.028$, $P < 0.01$), treatment ($F_{1,13} = 58.597$, $P < 0.01$) and treatment \times test phase interaction ($F_{4,52} = 9.012$, $P < 0.01$). More importantly, Lat A-mediated disappearance of morphine-induced CPP was persistent for more than 2 weeks compared with vehicle treatment, 3.29 ± 36.95 s vs. 238.06 ± 40.00 s (test 3), $P < 0.01$ by Tukey's post hoc test, and the morphine injection did not induce the reinstatement of morphine-induced CPP in Lat A-treated rats, 34.67 ± 35.52 s vs. 338.2 ± 31.15 s, $P < 0.01$ by Tukey's post hoc test (Fig. 4B). These results indicate that a single Lat A microinjection has a persistent effect on the disruption of morphine-induced CPP for more than 2 weeks.

Inhibition of actin polymerization in the NAc shell outside the reconsolidation window has no effect on morphine-induced CPP.

Previous studies showed that the time course of memory reconsolidation ranges from minutes to hours. However, the time window of morphine-induced CPP reconsolidation is unknown, which is essential for clinical applications. To further confirm that inhibition of actin polymerization in the NAc shell inhibits morphine-induced CPP by disrupting its reconsolidation and test the window of reconsolidation of morphine-induced CPP, the microinjection of Lat A was performed 6 hours after retrieval and morphine-induced CPP was tested on the next day (test 2) (Fig. 5A). Although statistical analysis of CPP scores revealed a significant effect of test phase ($F_{2,24} = 33.847$, $P < 0.01$), no significant difference between vehicle and Lat A treatments was detected ($F_{1,12} = 0.483$, $P > 0.05$) and no significant difference was revealed from the interaction between treatment and test phase ($P > 0.05$; Fig. 5B). These results demonstrate that Lat A treatment applied 6 hours after retrieval, which is outside the reconsolidation window, has no effect on the expression of morphine-induced CPP.

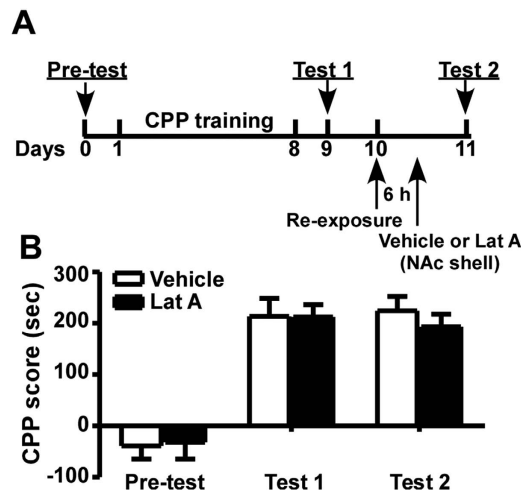


Figure 5. Inhibition of actin polymerization in the NAc shell outside the reconsolidation window has no effect on morphine-induced CPP. (A) Timeline of the experimental procedure. On day 10, the rats received vehicle or Lat A microinjections 6 hours after re-exposure to the morphine-paired chamber. (B) CPP scores of rats receiving microinjections into the NAc shell. Values represent mean \pm SEM, $n = 7-8$ /group.

Discussion

Relapse is a major challenge of drug addiction recovery. Even after prolonged abstinence, re-exposure to drug-related environmental cues can re-activate drug reward memory and elicit drug craving, leading to reinstatement of drug-seeking behavior, which is a major cause of failure in preventing relapse. Thus, it is necessary to develop a therapeutic approach to disrupt the associative memory between drug and drug-related cues. Recent studies suggest that memory reconsolidation plays a key role in contextual cue-induced relapse²⁹⁻³⁶. In addition, actin dynamics is implicated in memory consolidation and reconsolidation¹⁹. Moreover, accumulating evidence suggests that actin dynamics is implicated in drug addiction and reinstatement. For example, actin rearrangement is involved in drug-associated memories^{20,21}. Glutamate receptors, calcium/calmodulin-dependent protein kinase II (CAMKII), mammalian target of rapamycin (mTOR), Lim-kinase (LIMK) and Rho guanosine triphosphatases are involved in drug addiction and its treatment, which all negatively or positively affect actin rearrangements³⁷⁻⁴². Although the evidence suggests that manipulating actin dynamics may be a potential approach to disrupt cue-associated drug memories by affecting memory reconsolidation, no direct evidence confirm this hypothesis. In this study, we demonstrate that inhibiting actin polymerization disrupts morphine-induced CPP, mediated by disrupting the reconsolidation of associative memories between morphine and morphine-related cues. In addition, a single post-retrieval injection of Lat A has robust and long-term benefits to inhibit morphine-induced CPP, indicating that post-retrieval inhibition of actin polymerization is a potential approach to prevent relapse, which has a long lasting effect.

Although both the NAc shell and core are involved in drug addiction and drug memory reconsolidation, they do have distinct functions in these processes. Previous studies showed that the NAc core play an important role in drug memory reconsolidation. For example, infusion of the protein synthesis inhibitor into the NAc core disrupts the reconsolidation of cocaine-associated memory³¹. Recently, it has been reported that NAc shell Arc/Arg3.1 protein, but not the protein in the NAc core, mediates the reconsolidation of morphine-induced CPP⁴³. Our data first showed that disrupting actin polymerization in the NAc shell contributes to the impaired reconsolidation of morphine-induced CPP, while the NAc core is not involved in this process. The above evidence suggests that the NAc core and shell play distinct roles in drug memory reconsolidation depending on differential drugs (e.g., cocaine and morphine) and molecular pathways (e.g., protein synthesis, proteasome degradation and actin dynamics) involved.

Our data indicates that inhibiting actin polymerization in the NAc shell inhibits morphine-induced CPP mediated by disrupting drug memory reconsolidation. Young *et al.* showed that inhibiting actin polymerization in the basolateral amygdala (BLA) inhibits the consolidation of the methamphetamine-associated memory, but not the reconsolidation²⁰. It seems like that the effect of Lat A, the actin polymerization inhibitor, on drug memory is contradictory. However, two key points should be noted, different brain regions and different drugs. First, the NAc shell and BLA play differential roles in memory process, which may contribute to differential effects of Lat A on the memory formation, storage and maintenance⁴⁴. For example, the BLA is mainly involved in emotional modulation of memory, while the NAc plays a major role in drug reward memory^{31,43,45-48}. In addition, morphine and methamphetamine induce drug addiction through differential mechanisms, which may also contribute to differential effects of actin polymerization inhibition. For example, morphine indirectly induces dopamine release mediated

by opioid receptor activation, while methamphetamine directly increases dopamine level by inhibiting dopamine transporter-mediated dopamine reuptake and potentiating dopamine release^{43,49–53}.

In conclusion, we first demonstrate that actin polymerization in the NAc shell plays a pivotal role in the reconsolidation of morphine-induced CPP. Moreover, inhibition of actin polymerization abolishes morphine-induced CPP by disrupting its reconsolidation. Our work provides a novel insight that inhibition of actin polymerization is a potential approach to prevent relapse and the intervention should be applied within a limited time window after retrieval.

Materials and Methods

Subjects. Male Sprague-Dawley rats, weighing 230–250 g, were obtained from the Laboratory Animal Center, Peking University Health Science Center. They were housed four per cage in an animal facility with constant temperature ($23 \pm 2^\circ\text{C}$) and humidity ($50 \pm 5\%$). The rats access to water and food ad libitum under a reverse 12h light: 12h dark cycle. The experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Local Animal Care and Use Committee.

Drugs. Morphine sulfate (Qinghai Pharmaceutical Ltd, Xining, China) was dissolved in 0.9% physiological saline and injected subcutaneously (10 mg/kg) in a volume of 1 ml/kg before exposure to a drug-paired context during the CPP training sessions. Latrunculin A (Lat A) (Merck, 428021-100UG, Germany) was dissolved in DMSO ($25 \mu\text{g}/\mu\text{l}$) and diluted in PBS to a final concentration of $0.5 \mu\text{g}/\mu\text{l}$. The dose of Lat A was chosen based on the work by Hou *et al.*⁵⁴. The vehicle was PBS which contained 2% DMSO.

Surgery and intracranial injections. All surgical procedures were performed as previously described⁵⁵. Briefly, rats weighing 290–310 g were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and permanent guide cannulae (23 gauge; Plastics One, Roanoke, VA, USA) were implanted bilaterally 1 mm above the NAc shell and core. The stereotaxic coordinates for the NAc shell were anterior/posterior (AP), +1.8 mm, medial/lateral (ML), +3.2 mm (16° angle) and dorsal/ventral (DV), –6.6 mm. The stereotaxic coordinates for the NAc core were AP, +1.5 mm, ML, +3.8 mm (16° angle) and DV, –6.0 mm. Stainless steel screws and dental cement were used to anchor the cannulae to the skull. Each cannula was inserted by a stainless steel blocker to maintain patency and prevent infection. The rats were given 1 week to recover from surgery before the subsequent experiments were carried out.

Injections were carried out with Hamilton microsyringes, which were connected to 30-gauge injectors (Plastics One, USA). Lat A ($0.5 \mu\text{g}/\mu\text{l}/\text{side}$) or vehicle ($0.5 \mu\text{l}/\text{side}$) was infused bilaterally into the NAc core and shell (Fig. 1), respectively, at a rate of $0.5 \mu\text{l}/\text{min}$. The injectors were kept in place for another 2 minutes to allow the drug to completely diffuse to the target region.

Conditioned place preference (CPP). Morphine-induced CPP was carried out using an unbiased, counterbalanced protocol. CPP training procedures were described previously^{25,56}. Briefly, the CPP apparatus consists of nine identical three-chamber polyvinyl chloride (PVC) boxes. In each box, two large chambers (27.9 cm long \times 21.0 cm wide \times 20.9 cm high) are separated by a smaller chamber (12.1 cm long \times 21.0 cm wide \times 20.9 cm high). The three distinct chambers were divided by manual guillotine doors. The floor texture of the two large chambers (bar or grid, respectively) was different from each other to provide distinct visual cues paired with morphine or saline injections.

In the baseline place preference test (preconditioning test, pre-test), the rats were initially placed in the center chamber and allowed ad libitum access to the three chambers for 15 minutes. The time spent in the designated morphine- or saline-paired chamber during the 15-minute session was recorded by detecting infrared beam breaks. Twenty-one rats showing a strong unconditioned preference (> 540 s) for one compartment were excluded. During the conditioning days, the rats were trained for 8 consecutive days with alternative injections of morphine (10 mg/ml/kg, s.c.) or saline (1 ml/kg, s.c.) in the designated chambers. After each injection, the rats were placed in the morphine- or saline-conditioned chamber for 45 minutes before brought back to their home cages. One day after the last conditioning trial, the expression of morphine-induced CPP was tested under conditions identical to those described in the baseline preference test. The CPP score was defined as the difference in time (in seconds) spent in the morphine-paired and saline-paired chambers during CPP testing.

Experimental design. Experiment 1: Two groups of rats were ($n=7-8$ per group) trained for morphine-induced CPP for 8 days. On day 9, all rats were tested for morphine-induced CPP (test 1). On day 10, all rats were re-exposed to the morphine-paired chamber for 10 minutes to reactivate morphine-associated memory, referred as reactivation or retrieval. Two groups of rats received bilateral Lat A ($0.5 \mu\text{g}/\mu\text{l}$) or vehicle ($0.5 \mu\text{l}$) microinjections into the NAc core immediately after the reactivation, respectively. On day 11, all rats were retested for morphine-induced CPP (test 2).

Experiment 2: Four groups of rats ($n=7-8$ per group) were trained for morphine-induced CPP for 8 days. On day 9, all rats were tested for morphine-induced CPP (test 1). On day 10, two groups of rats were re-exposed to the morphine-paired chamber for 10 minutes and received bilateral Lat A ($0.5 \mu\text{g}/\mu\text{l}$) or vehicle ($0.5 \mu\text{l}$) microinjections into the NAc shell immediately after the reactivation. In

the meantime, the other two groups of rats without reactivation received bilateral Lat A (0.5 µg/µl) or vehicle (0.5 µl) microinjections into the NAc shell, respectively. On day 11, all four rats were retested for morphine-induced CPP (test 2).

Experiment 3: Two groups of rats (n = 7–8 per group) were trained for morphine-induced CPP for 8 days. On day 9, all rats were tested for morphine-induced CPP (test 1). On day 10, two groups of rats were re-exposed to the morphine-paired chamber for 10 minutes and received bilateral Lat A (0.5 µg/µl) or vehicle (0.5 µl) microinjections into the NAc shell immediately after the reactivation. On day 11 and 25, all rats were retested for morphine-induced CPP (test 2 and test 3). On day 26, both vehicle and Lat A-treated rats received a priming injection of morphine (3 mg/ml/kg, s.c.), and the CPP test was immediately performed after the priming injection (priming test).

Experiment 4: Two groups of rats (n = 7–8 per group) were trained for morphine-induced CPP for 8 days. On day 9, all rats were tested for morphine-induced CPP (test 1). On day 10, two groups of rats were re-exposed to the morphine-paired chamber for 10 minutes and received bilateral Lat A (0.5 µg/µl) or vehicle (0.5 µl) microinjections into the NAc shell 6 hours after the reactivation. On day 11, all rats were retested for morphine-induced CPP (test 2).

Statistical analysis. Values are expressed as mean ± SEM. The data were analyzed by two-way repeated-measures analysis of variance (ANOVA) with the between-subjects factor of treatment (vehicle or Lat A) and within-subjects factor of test phase, followed by Tukey's post hoc test. $p < 0.05$ was accepted as statistical significance.

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Author Contributions

G.L. and H.M. conceived and designed the experiments. Y.Wang, M.Y., Y.X., X.S. and Q.L. performed the experiments. G.L. and Y.Wu analyzed the data and wrote the paper. All authors reviewed the manuscript.

Additional Information

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